PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/11, 15/90, 15/82
C12N 5/10, A01H 5/00

A1

(11) International Publication Number: WO 93/07266

(43) International Publication Date: 15 April 1993 (15.04.93)

(21) International Application Number: PCT/US92/08513

(22) International Filing Date: 6 October 1992 (06.10.92)

(30) Priority data:
773,333 7 October 1991 (07.10.91) US

(60) Parent Application or Grant
(63) Related by Continuation
US 07/773,333 (CIP)
Filed on 7 October 1991 (07.10.91)

(71) Applicant *(for all designated States except US):* IDAHO RESEARCH FOUNDATION, INC. [US/US]; 121 Sweet Avenue, Moscow, ID 83843-0178 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only) :* GUERRA, Daniel, J. [US/US]; Route 1, Box 50, Troy, ID 83871 (US). XIANG, Chengbin [CN/US]; 23 Park Village, Moscow, ID 83843 (US).

(74) Agents: STEPHENS, Donald, L., Jr. et al.; Klarquist, Sparkman, Campbell, Leigh & Whinston, One World Trade Center, Suite 1600, 121 S.W. Salmon Street, Portland, OR 97204 (US).

(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CS, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, PL, RO, RU, SD, SE, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: GENETIC CONSTRUCT FOR SELECTION OF HOMOLOGOUS RECOMBINANTS ON A SINGLE SELECTIVE MEDIUM

(57) Abstract

Novel genetic constructs and methods for their use in transforming target cells are disclosed. The genetic constructs, which are particularly adapted for homologous recombination with target-cell genomic DNAs, comprise a positively selectable genetic marker and a negative selection system "antagonistic" to the expression of the positively selectable marker. The positively selectable marker is situated in a region of the construct between a first and a second flanking sequence homologous to sequences flanking a desired integration site in the target-cell genome. The negative selection system is situated outside the region. The negative selection system preferably comprises an antisense gene that prevents expression of the positively selectable marker. The positively selectable marker preferably encodes an antibiotic resistance factor. The construct can also include a "gene of interest", also situated between the homologous flanking sequences adjacent the positively selectable marker, for introduction via homologous recombination into the target genome at the desired integration site. Transformed target cells whose genomes homologously recombine with the genetic construct acquire the positively selectable marker and (if present) the gene of interest. Non-homologous recombinants acquire both the positively selectable marker and the negative selection system, thereby losing the ability to survive exposure to the corresponding positive selection agent. Non-recombinants acquire no part of the construct. Thus, homologous recombinants have the exclusive ability to survive exposure to the positive selection agent and can be easily isolated from all other target cells.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**FR**	France	**MR**	Mauritania
AU	Australia	**GA**	Gabon	**MW**	Malawi
BB	Barbados	**GB**	United Kingdom	**NL**	Netherlands
BE	Belgium	**GN**	Guinea	**NO**	Norway
BF	Burkina Faso	**GR**	Greece	**NZ**	New Zealand
BG	Bulgaria	**HU**	Hungary	**PL**	Poland
BJ	Benin	**IE**	Ireland	**PT**	Portugal
BR	Brazil	**IT**	Italy	**RO**	Romania
CA	Canada	**JP**	Japan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SK**	Slovak Republic
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SN**	Senegal
CM	Cameroon	**LK**	Sri Lanka	**SU**	Soviet Union
CS	Czechoslovakia	**LU**	Luxembourg	**TD**	Chad
CZ	Czech Republic	**MC**	Monaco	**TG**	Togo
DE	Germany	**MG**	Madagascar	**UA**	Ukraine
DK	Denmark	**ML**	Mali	**US**	United States of America
ES	Spain	**MN**	Mongolia	**VN**	Viet Nam
FI	Finland				

GENETIC CONSTRUCT FOR SELECTION OF HOMOLOGOUS RECOMBINANTS ON A SINGLE SELECTIVE MEDIUM

FIELD OF THE INVENTION

This invention pertains to the fields of recombinant DNA technology, transformation of cells, and genetic selection of transformed cells.

BACKGROUND OF THE INVENTION

Gene Regulation by Antisense

A principal scheme by which information is transferred from a DNA sequence is, first, by transcription of the DNA sequence to form a corresponding RNA message ("messenger RNA" or "mRNA"), then by translation of the mRNA to form a corresponding protein. This seemingly simple scheme occurs effected by complex mechanisms in living cells. This scheme is further complicated by the fact that not all genes in a cell's genome are expressed at the same time. Cells possess complex regulatory mechanisms that selectively turn genes on and off, thereby determining cell structure, function, and developmental fate.

Certain classes of DNA sequences in cells are termed "regulatory genes" because they regulate the expression of other DNA sequences. One class is comprised of regulatory genes encoding regulator proteins that interact with other specific DNA sequences at the transcriptional or translational level to modulate the amount of protein produced by the regulated DNA sequence, often in response to environmental cues. Other classes of regulatory genes function by means other than by producing a regulatory protein.

Mounting evidence indicates that an important naturally occurring means of gene regulation is by "antisense" transcripts, particularly in procaryotic cells. Tomizawa et al., Proc. Natl. Acad. Sci. USA 78:1421-1425 (1981); Mizuno et al., Proc. Natl. Acad. Sci. USA 81:1966-1970 (1984); and Simons, Gene 72:35-44 (1988). An antisense transcript, termed "antisense RNA," is generally comprised of a nucleotide sequence

complementary to either an mRNA encoded by a regulated DNA sequence or to the "sense strand" of DNA comprising the regulated sequence. One probable mechanism by which an antisense RNA regulates a DNA sequence is by the formation of a hybrid RNA duplex of the antisense RNA with a particular mRNA via Watson-Crick base pairing. Van der Krol et al., Gene 72:45-50 (1988). Such hybrids appear to be resistant to translation for any of a number of reasons including unusually rapid degradation of the duplex in the cell, impairment of post-transcriptional processing, or inhibition of ribosome binding. Simons, Gene 72:35-44 (1988); and Van der Krol et al., Gene 72:45-50 (1988). DNA sequences that encode antisense RNAs are termed "antisense genes" or "antisense DNA sequences."

After the discovery of antisense RNAs, researchers began investigating ways to artificially regulate gene expression using antisense RNAs. These studies have proven effective in identifying specific DNA sequences, characterizing the function of particular DNA sequences, controlling infections, and manipulating metabolic pathways. Van der Krol et al., BioTechniques 6:958-976 (1988). For example, antisense RNA synthesized in vitro and introduced into eucaryotic cells can regulate expression of specific DNA sequences within the cells, at least temporarily. Izant and Weintraub, Cell 36:1007-1015 (1984); Izant and Weintraub, Science 229:345-352 (1985); and Van der Krol et al., BioTechniques 6:958-976 (1988). These experiments have not only been performed using mammalian cells but also plant cells. Van der Krol et al., Gene 72:45-50 (1988); Smith et al., Nature 334:724-726 (1988); Sheehy et al., Proc. Natl. Acad. Sci. USA 85:8805-8809 (1988); Van der Krol et al., Nature 333:866-869 (1988); Ecker et al., Proc. Natl. Acad. Sci. USA 83:5372-5376 (1986); and Rothstein et al., Proc. Natl. Acad. Sci. USA 84:8439-8443 (1987). Simultaneous development of DNA and RNA sequencing, DNA cloning, and in vitro DNA-

and RNA-synthesis technology made it possible to readily generate antisense RNAs to target DNA sequences. Inouye, Gene 72:25-34 (1988).

Antisense RNA can be used to mimic mutations in both procaryotic and eucaryotic organisms. Takayama and Inouye, Crit. Rev. in Biochem. and Molec. Biol., CRC Press 25:155-184 (1990); and van der Krol et al., BioTechniques 6:958-976 (1987). In plants, antisense RNA has been successfully used to inhibit the activity of nopaline synthase, Rothstein et al., Proc. Natl. Acad. Sci. USA 84:8439-8443 (1987), and Sandler et al., Plant Mol. Biol. 11:301-310 (1988); chloramphenicol acetyltransferase, Ecker and Davis, Proc. Natl. Acad. Sci. USA 83:5372-5376 (1986), and Delauney et al., Proc. Natl. Acad. Sci. USA 85:4300-4304 (1900); chalcone synthase, van der Krol et al., Nature 333:866-869 (1988); polygalacturonase, Smith et al., Nature 334:724-726 (1988), and Sheehy et al., Proc. Natl. Acad. Sci. USA 85:8805-8809 (1988); β-glucuronidase, Robert et al., Plant Mol. Biol. 13:399-409 (1989); and granule-bound starch synthase, Visser et al., Mol. Gen. Genet. 225:289-296 (1991).

Because mRNAs have only a limited life in cells, antisense RNA injected into cells generally exhibits a regulatory function of relatively short duration. However, functional antisense DNA sequences can be made in vitro by coupling a functional promoter to a DNA sequence oriented in a way wherein the promoter induces transcription of the noncoding (or "nonsense") strand of the DNA, rather than the normal coding (or "sense") strand. One approach is to invert the coding sequence of the DNA relative to the promoter. Izant and Weintraub, Science 229:345-352 (1985). This can be done by excising the coding region, proximal to the promoter and polyadenylation sites, and reinserting the excised portion in reverse orientation relative to the promoter. Izant and Weintraub, Cell 36:1007-1015 (1984). Thus, the antisense DNA sequence is transcribed in a direction

opposite to the direction of transcription of the corresponding sense DNA sequence. Mizuno et al., Proc. Natl Acad. Sci USA 81:1966-1970 (1984). When such an antisense sequence is introduced into a recipient cell having an endogenous corresponding "sense" DNA sequence, the promoter directs transcription of the nonsense DNA strand, producing a transcript (antisense RNA) complementary to mRNAs normally produced by the corresponding "sense" sequence. Introduction of an antisense DNA sequence into a cell has been found to result in a more prolonged, or stable, regulatory effect than introduction of antisense RNA, so long as the introduced antisense DNA sequence continues to be transcribed by the cell. Of course, integrating the introduced antisense DNA sequence into the cell genome can result in continued expression of the antisense sequence over the lifetime of the cell.

Many plant cells, in contrast to the vast majority of other eucaryotic cells, are totipotent. That is, individual totipotent plant cells can be readily induced to divide and form entire plants. Hence, introduction of an exogenous antisense regulatory DNA sequence into a totipotent plant cell, wherein the antisense sequence integrates into the cellular genome, can yield a source of "genetically engineered" plants expressing the new antisense sequence in their cells and passing the antisense DNA sequence to their progeny. Rothstein et al., Proc. Natl. Acad. Sci. USA 84:8439-8443 (1987).

Homologous Recombination

When an exogenous DNA sequence is introduced into a cell, the introduced sequence can become integrated into the cell genome. Random integrations are inefficient in producing specific desired genetic alterations. However, it is now possible to "target" the integration to a specific site in the cell genome by a technique known as homologous recombination. See, e.g., Capecchi,

Trends in Genet. 5:70-76 (1989); and Capecchi, Science 224:1288-1292 (1989).

When homologous recombination results in insertion of the introduced DNA sequence into the cell genome, the inserted DNA is placed under normal endogenous controls by which expression of genes is regulated in the cell. Thus, the introduced sequence can be employed to repair a genetic lesion in the cell, impart a modified phenotype to the cell, or disrupt a preexisting allele in the cell.

In general, homologous recombination has been utilized for insertion of exogenous and other genetic sequences into cellular genomes, for replacement of endogenous genome sequences with introduced sequences, and for inducing mutations in a particular endogenous DNA sequence by "heteroduplex-induced mutagenesis" resulting from incorrect repair of a transient heteroduplex that forms between the newly introduced DNA and the homologous chromosomal sequence during the homologous recombination event. Thomas and Capecchi, Cold Spring Harbor Symp. on Quant. Biol. 51:1101-1113 (1986); and Thomas and Capecchi, Cell 51:503-512 (1987).

Specific examples of homologous recombination include the following:

(a) Creation of a large deletion at the T-cell antigen receptor β-subunit locus in mouse embryo stem cells. Mombaerts et al., Proc. Natl. Acad. Sci. USA 88:3084-3087 (1991).

(b) Introduction of mutations into the hox-2.6 locus, Hasty et al., Nature 350:243-246 (1991); the hox-1.5 locus, Chisaka et al., Nature 350:473-479 (1991); the hox-1.3 locus, Jeannotte et al., Mol. Cell Biol. 11:5578-5585 (1991); and the hox-1.6 locus, Chisaka et al., Nature 355:516-520 (1992), of mouse embryonic stem cells.

(c) Introduction of a null mutation into the p53 tumor-suppressor gene to investigate possible roles of

this gene in mammalian tumorigenesis. Donehower et al., Nature 356:215-221 (1992)).

Gene targeting involving homologous recombination has also been described in other eucaryotic systems including human cells, Smithies et al., Nature 317:230-234 (1985); yeast, Hinnen et al., Proc. Natl. Acad. Sci. USA 75:1929-1933 (1978); Dictyostelium, De Lozanne et al., Science 236:1086-1091 (1987); Trypanosoma brucei, Lee et al., Science 250:1583-1587 (1991); and Aspergillus parasiticus, Horng et al., Mol. Gen. Genet. 224:294-296 (1990).

Gene targeting has also been utilized in plants. Gal et al., EMBO J. 10:1571-1578 (1991); Pazkowski et al., EMBO J. 7:4021-4026 (1988); Offringa et al., EMBO J. 9:3077-3084 (1990); Peterhans et al., EMBO J. 9:3437-3445 (1990); Lee et al., Plant Cell 2:415-425 (1990); Lynik et al., Mol. Gen. Genet. 230:209-218 (1991); and Halfter et al., Mol. Gen. Genet. 231:186-193 (1991). In this work, defective selectable marker genes were stably integrated into the nuclear genomes of subject plant cells by using either direct (e.g., via electroporation or microinjection) or Agrobacterium-mediated gene transfer. Subsequently, "correct" exogenous DNA sequences were introduced into the plant cells harboring the previously introduced defective markers, resulting in a restored selectable marker via homologous recombination. Gene targeting by homologous recombination has also been used to disrupt chloroplast genes in unicellular photosynthetic organisms. Takahashi et al., EMBO J. 10:2033-2040 (1991): Smart et al., EMBO J. 10:3289-3296 (1991); and Newman et al., Mol. Gen. Genet. 230:65-74 (1991).

Gene targeting requires that a highly discriminating procedure be utilized to isolate cells that have undergone the desired homologous recombination. The frequency of transgene homologous recombination is reportedly about one in 10^3 to about one in 10^5. Lee et al., Plant Cell 2:415-425 (1990). All

gene targeting experiments performed to date with higher plants, including experiments reported in the references cited in the previous paragraph, involved the introduction into subject plant cells of positively-selectable marker genes. While these experiments demonstrated that gene targeting in higher plants was possible, they also showed that, even with positively-selectable markers, exhaustive screening techniques such as Southern blotting or PCR techniques were required in order to distinguish and isolate the relatively very few homologously recombinant cells that were formed from other recombinants and from non-recombinants. Capecchi, Science 244:1288-1293 (1989). The experiments also indicated that gene targeting in plants, using existing methods, could not be readily performed at all using nonselectable genes or DNA sequences that do not possess a selectable marker. In addition, there are no practical or reliable negatively selectable markers for plant systems. These problems pose a major barrier for the application of gene targeting in higher plants.

As with plant cells, there is also a need for a practical way to distinguish and isolate homologously recombinant animal cells from a population comprised of homologous recombinants, non-homologous recombinants, and non-recombinants. Existing methods for animal cells are difficult, time-consuming, and/or expensive to perform, requiring either multiple selection agents and/or exhaustive screening of the entire population of cells.

Hence, there is a need for a practical way, after introducing genetic constructs into cells to undergo homologous recombination with the cellular genomes, for identifying and isolating the desired homologous recombinants from the entire population of transformed and non-transformed cells.

There is also a need for genetic constructs capable of undergoing homologous recombination with the genomes of target cells which confer upon the resulting

homologous recombinants the ability to be selected for using only one positive selection agent, thereby permitting the desired homologous recombinants to be readily recovered from other cells that are either non-transformed or non-homologously transformed.

SUMMARY OF THE INVENTION

The aforementioned needs are met by the present invention which provides genetic constructs capable of homologous recombination with an integration site in the DNA of target cells. The genetic constructs are particularly adapted for introducing new genetic information or mutations at specific site(s) in the DNA of target cells while enabling only those target cells that have acquired the new information or mutation at the desired site(s) to be readily isolated from all other cells. Thus, expensive and time-consuming screening procedures are obviated because the previously mandatory need to screen all the target cells is eliminated.

A genetic construct according to the present invention comprises a positively selectable genetic marker (i.e., a DNA sequence encoding a positively selectable genetic trait) that integrates into the cell DNA at the integration site during recombination events between the construct and the cell DNA. The genetic construct also comprises a negative selection system "antagonistic" to expression of the positively selectable genetic marker. The negative selection system is integratable into the cell DNA via non-homologous recombination between the construct and the cell DNA but not via homologous recombination.

Genetic constructs according to the present invention confer only upon homologously recombinant cells the ability to survive exposure to a single positive selection agent (to which the positively selectable genetic marker confers resistance); non-recombinants and non-homologous recombinants are

incapable of surviving exposure to the positive selection agent.

The positively selectable genetic marker preferably, but not necessarily, encodes an antibiotic-resistance factor. The negative selection system is preferably comprised of an antisense DNA sequence of the positively selectable marker. Thus, for example, in non-homologous recombinants receiving both the DNA sequence encoding the antibiotic-resistance factor and the antisense DNA sequence, the antisense DNA sequence blocks expression of the antibiotic-resistance sequence, rendering the non-homologous recombinant target cell incapable of surviving exposure to the corresponding antibiotic. Also, since non-recombinants never acquire the antibiotic-resistance sequence, they are also killed by the antibiotic. Homologous recombinants, on the other hand, integrate the DNA sequence for antibiotic resistance but do not integrate the antisense sequence. Therefore, homologous recombinants are the only cells that survive exposure to the antibiotic.

Selective integration of the positively selectable genetic marker (but not the negative selection system) at the desired site in the target cell DNA is made possible by placing the positively selectable marker inside a region bounded by flanking sequences homologous to genetic sequences flanking the desired integration site in the cell DNA. The negative selection system, however, is located outside the region bounded by the homologous flanking sequences. Thus, the negative selection system is not integrated into the cell DNA via a homologous recombination event.

Genetic constructs according to the present invention can also include a "gene of interest" (i.e., any desired DNA sequence) located adjacent the positively selectable marker sequence between the homologous flanking sequences. Target cells into which such a construct is introduced integrate not only the positively selectable marker but also the gene of

interest as a result of a homologous recombination event. The gene of interest can be an "improved" DNA sequence from the target-cell organism, a DNA sequence from a different type of organism, a group of DNA sequences, or a portion of a gene or genes. Thus, it is now possible to introduce specific genetic changes at specific sites in cell DNA without having to laboriously screen all target cells for "proper" recombinants.

If the target cells have totipotency, it is now also possible to impart desired genetic changes in such target cells, rapidly and inexpensively screen the putative homologous recombinants, and generate genetically identical entire organisms from the "proper" recombinants. Because many plant cells are totipotent and can be readily induced to develop into new plants, the present invention has unlimited benefit in the "genetic engineering" of improved varieties of plants.

A general requirement of the target cells is that they be able to express the genetic information supplied to the cells in the genetic constructs of the present invention. Thus, if the negative selection system comprises an antisense DNA sequence to the positively selectable marker sequence, it is necessary that the target cell be able to express the antisense sequence. Accordingly, the positively selectable marker and the negative-selection sequence must each be in the proper "sense" and be controlled by a promoter and a terminator expressible in the target cells.

The genetic constructs according to the present invention can be constructed using any of the well-developed methods used in the art for rearranging, cloning, and assaying new genetic constructs. Likewise, they can be introduced into target cells by any of a variety of methods known in the art, including using a vector delivered by an infectious agent, electroporation, or other technique.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a generic scheme of a genetic construct according to the present invention.

FIG. 2 is a representative embodiment of the construct of FIG. 1 particularly suitable for transforming plant cells.

FIG. 3 shows genetic maps of the genetic constructs of Examples 1-4.

FIG. 4 shows the results of the transient-expression assays of Examples 5-6 wherein pBSIIKS-/npt+ and pBSIIKS-/npt- plasmids were electroporated in protoplasts to determine the effect of the anti-nptII gene on nptII gene expression.

FIG. 5 shows the results described in Examples 7-11 involving biological assays of the antisense genetic constructs of Examples 1-4, wherein the presence of any of said antisense constructs in leaf cells renders the cells incapable of forming calli and shoots.

FIG. 6 schematically shows the pCX407/hpt and pKYLX7 constructs described in Examples 18-19.

FIG. 7 shows Southern blots of total DNA from kanamycin-sensitive callus tissue indicating the presence of both nptII and anti-nptII genes, as described in Examples 20-24.

FIG. 8A is a Northern blot of total RNA from transformed and control calli probed with a digoxigenin-labeled "sense" nptII sequence, as described in Examples 25-29.

FIG. 8B is a Northern blot of total RNA from transformed and control calli probed with a digoxigenin-labeled "antisense" nptII sequence, as described in Examples 25-29.

FIG. 9 shows genetic maps of alternative embodiments of constructs according to the present invention particularly suitable for transforming plant cells, as described in Example 30.

DETAILED DESCRIPTION

According to one aspect of the present invention, genetic constructs are provided that, in general, are capable of homologous recombination with genomic DNA of target cells so as to introduce new DNA sequences into the cells' genomic DNA. Such genetic constructs are referred to as being capable of "transforming" target cells of interest. (As used herein, "transformation" is a process by which naked DNA is introduced into a cell so as to cause a heritable change to the cell.) The genetic constructs have particular utility for use in transforming cells by homologous recombination, wherein it is desired that only homologous recombinants, not non-homologous recombinants or non-recombinants, be recovered. The present invention makes it possible, for the first time, to recover homologous recombinants by a simple, easy, and inexpensive selection procedure requiring only a single selective medium.

Each genetic construct, as shown generally in FIG. 1, comprises both a positive selection system and a negative selection system. The positive selection system comprises a positively selectable marker (PSM) locus such as, but not limited to, a locus encoding an antibiotic resistance factor. The negative selection system comprises a locus "antagonistic" to the PSM locus, i.e., the negative selection system blocks expression of the PSM locus. A preferred type of negative selection system comprises an "antisense locus" (AS_{PSM} locus) encoding an antisense RNA capable of blocking expression of the PSM locus.

The AS_{PSM} locus exercises its blocking function against the PSM locus by any of several antisense mechanisms known in the art, including inhibition of transcription (e.g., by binding to DNA encoding the PSM locus), by binding to mRNA transcribed from the PSM locus before the mRNA is translated, or by otherwise interfering with the translation machinery in the target cell (e.g., binding to ribosomes). The present state of

knowledge pertaining to antisense action indicates that inhibition of translation is the predominant general mechanism of antisense RNA action.

Optionally included in the genetic construct is a "gene of interest" (GOI) locus encoding one or more genes (which can be non-selectable DNA sequences), or a portion of a gene, intended to be incorporated by homologous recombination into the genomes of recipient, or "target," cells. If present, the GOI locus is situated adjacent the PSM locus.

The PSM locus, and the GOI locus if present, are situated between homologous genomic flanking sequences designated "HFS_1" and "HFS_2" in FIG. 1. The HFS_1 and HFS_2 sequences are homologous to genomic sequences normally flanking the 5′ and 3′ ends of a desired integration site in the cell genome. For example, if the desired integration site is a particular target DNA sequence, the HFS_1 and HFS_2 sequences are homologous to genomic sequences flanking the target sequence. As has been established in the research literature, homologous flanking sequences on a length of DNA introduced into a cell to transform the cell substantially increase the probability that the DNA extending between the homologous flanking sequences will integrate at the desired site in the target cell genome. Thomas and Capecchi, Cold Spr. Harb. Symp. Quant. Biol. 51:1101-1113 (1986).

As shown in FIG. 1, the AS_{PSM} locus is situated on the construct outside the region flanked by HFS_1 and HFS_2. This ensures that the AS_{PSM} locus will not become integrated into the target cell genome as a result of a desired homologous recombination event, but will likely become integrated if the construct integrates into the target genome via non-homologous recombination. Non-recombinants acquire neither the PSM locus nor the AS_{PSM} locus. Thus, homologous recombinants acquire the PSM locus conferring an ability to survive in a single selective medium but do not acquire the AS_{PSM} locus

antagonistic to expression of the PSM locus. Since non-homologous recombinants acquire both loci, they lack an ability to survive in the single selective medium. Because non-recombinants acquire neither locus, they also lack an ability to survive in the single selective medium. As a result, only homologous recombinants survive.

If the GOI locus comprises an entire gene intended to be introduced into a target cell to replace a corresponding defective gene in the cell genome, the HFSs are preferably homologous to sequences present on each side of the defective gene in the cell genome. If the GOI comprises only a portion of a gene, the HFSs can be homologous to other portions of the same gene adjacent the portion of the gene comprising the GOI.

The GOI locus (if present), PSM locus, and AS_{PSM} locus must be present in the genetic construct in the proper sense. In addition, the PSM and AS_{PSM} loci, and usually the GOI locus (if present) must be served by a suitable transcription promoter, designated in FIG. 1 as P1, P2, and P3, respectively. (As used herein, a "suitable" promoter is one that will function in the target cell and that will promote transcription of the corresponding locus.) But, the promoters need not be different; for example, the GOI and PSM loci can be controlled by the same promoter (i.e., P1 and P2 need not be different promoters). In addition, the PSM and AS_{PSM} loci, and usually the GOI locus (if present), must include a suitable transcription terminator, designated in FIG. 1 as T1, T2, and T3, respectively. If the GOI locus is only a portion of a complete gene of interest and is intended to impart a localized mutation to the target DNA sequence, there may be instances in which it is not necessary for the GOI to have a promoter or terminator.

It is preferred that P3, the antisense promoter, be a stronger promoter than P2, the PSM promoter, for effective inhibition of PSM expression by AS_{PSM}

transcripts. See, van der Krol et al., Nature 333:866-869 (1988). A strong P3 ensures a high production of antisense RNA relative to PSM transcripts. This is important because previous work has shown that there is a gene-dosage effect of antisense RNA regulation. Inouye, Gene 72:25-44 (1988). Alternatively, an increase in antisense RNA production, relative to PSM production, can be achieved by incorporating into the construct of FIG. 1 multiple copies of AS_{PSM}. Id.

The following are representative examples of suitable promoters for genetic constructs according to the present invention. These examples, however, are not in any way intended to be limiting because the research literature has many representative examples of promoters that could be used and that are well within the skill of practicing artisans to utilize in assembling a genetic construct according to the present invention. One example of a suitable PSM promoter (P2) is the relatively weak NOS (nopaline synthase) promoter; a strong antisense promoter (especially relative to the NOS promoter and particularly adapted for genetic constructs intended for transforming plant cells) is the 35S promoter from cauliflower mosaic virus (CaMV). The 35S promoter has the additional benefit of being a "constitutive" promoter, meaning that it is expressible in a wide variety of systems. An alternative AS promoter is the moderately strong PAL gene promoter. Other promoters include the "rubisco" promoter, the chlorophyll AB binding protein (CAB) promoter, and heat-shock promoters. A rule of thumb to keep in mind is that the AS promoter should be stronger than the PSM promoter to ensure relatively high expression of the antisense RNA.

Under certain instances, it is advantageous to use regulable promoters, particularly if it is important to be able to turn expression of the corresponding gene on and off or to modulate expression of the gene at will. Examples of regulable promoters include, but are not

limited to, inducible promoters (such as heat-shock promoters), repressible promoters, polymerase-specific promoters, development-specific promoters, and tissue-specific promoters.

As stated above, the GOI is a DNA sequence that can comprise a single gene, plural genes, or one or more portions of a gene or genes. For example, the GOI can comprise an entire structural gene (such as a gene encoding a novel enzyme) not normally present or expressed in the target cells. As further examples, and in accordance with a voluminous research literature showing the introduction of a wide variety of "foreign" genes into cells, the GOI can comprise a procaryotic structural gene to be transferred into plant cells or a plant gene to be transferred into mammalian cells or a mammalian gene to be transferred into plant cells. Alternatively, the GOI can comprise a portion of a gene, in which instance the construct that includes the gene portion can be used to impart site-specific mutagenesis of a corresponding gene in the target-cell genome. In fact, such a gene portion can be as small as a single nucleotide.

The target cells can be plant cells or animal cells. In fact, there is no known limit to the types of target cells transformable by genetic constructs according to the present invention. This is further evidenced by the fact that, as supported by a voluminous research literature, artificially introduced "foreign" antisense DNA sequences can function in a wide variety of recipient cell types. Therefore, it can be stated with confidence that constructs according to the present invention containing an AS_{PSM} locus will function in any recipient cell capable of expressing antisense DNA sequences. In addition, the method by which the constructs are delivered inside the target cells can be any of the several methods known in the art including, but not limited to, delivery by an infectious-agent vector (such as by Agrobacterium infection of target

plant cells), electroporation, particle-gun bombardment, and other suitable methods.

Depending upon the transformation method and the type of target cell, genetic constructs according to the present invention can have additional features. To illustrate, if the construct is used to transform plant cells, it can include features normally found on plasmids conventionally used to transform plant cells. For example, transformation of plant cells is commonly performed using a plasmid vector derived from T-DNA (virulence plasmid) of Agrobacterium tumefaciens. These plasmids include "right border" (RB) and "left border" (LB) regions that facilitate transformation; that is, the RB and LB regions increase the frequency of transformation of the target plant cells. It will be appreciated that, if the construct is used to transform animal cells, RB and LB regions are not necessary and can be, if desired, replaced with other regions that enhance transformation of animal cells.

A genetic construct according to the present invention is constructed by techniques within the knowledge and experience of persons skilled in the art. That is, the methodology of constructing in vitro novel combinations of genetic elements is well within the purview of persons skilled in the art armed with an arsenal of restriction enzymes and familiar with gene cloning techniques. These techniques are extensively detailed, for example, in Sambrook et al., Molecular Cloning, A Laboratory Manual, 2d ed. (3 volumes), Cold Spring Harbor Laboratory (1989). Hence, it will be appreciated that the PSM locus can comprise any of a variety of known clonable positive selectable markers, such as any of various antibiotic resistance markers described in the literature. Likewise, the GOI can be virtually any gene or gene fragment of interest, including any of the thousands described in the research literature. Since the PSM locus is clonable, it is a routine matter to generate an antisense locus

corresponding to the PSM locus. Inouye, Gene 72:25-34 (1988). Linking of the elements comprising a genetic construct according to the present invention is also within the purview of persons skilled in the art, employing the standard arsenal of restriction enzymes and using conventional separation and assay techniques.

Selecting proper HFS_1 and HFS_2 loci to include in a genetic construct according to the present invention is also within the purview of persons skilled in the art. Typically, molecular genetic details of the GOI will be known, including restriction enzyme cleavage maps and even an actual sequence of the GOI together with flanking sequences. (Knowledge of the actual nucleotide sequence of the GOI and flanking sequences is not required if suitable restriction fragments are available.)

The length of the HFS_1 and HFS_2 loci can vary. It will be appreciated by persons skilled in the art that increasing the length of the HFSs (within certain limits) will correspondingly improve the likelihood that the genetic construct will "find" the desired integration site in the target cell genome and successfully undergo recombination at that site. That is, increasing the length of the HFSs can yield a corresponding increase in the efficiency of successful recombination. Based on homologous recombination studies reported in the research literature performed using mammalian cells, and on our own research, an effective lower limit of the combined length of HFS_1 and HFS_2 is about 0.2 kilobases (kb). A reasonable upper limit is about 4.0 kb. The preferred range is between about 2.0 kb and 4.0 kb. It will also be appreciated that the total HFS length may have to be limited due to practical upper limits on the size of the genetic construct according to the present invention that can be delivered into a particular type of cell undergoing transformation. That is, the lengths of the GOI, PSM, AS_{PSM}, and other loci on the construct, relative to the

maximal allowable length of the construct, may demand that the HFSs be shorter than optimal.

The length of the AS_{PSM} locus will depend in part upon the length of the PSM. However, the AS_{PSM} locus need not be so long as to produce an antisense RNA having the same length as the PSM transcript. The length of the AS_{PSM} locus will also depend in part upon the mechanism of inhibitory action of the particular antisense RNA encoded by the AS_{PSM} locus selected; e.g., whether the antisense RNA acts by binding directly to coding regions of PSM mRNA so as to directly inhibit translation of the mRNA, whether the antisense RNA acts by binding to noncoding regions of PSM mRNA so as to inhibit translation, or whether the antisense RNA interferes in some way at the transcriptional level. A longer antisense RNA is not necessarily better than a short antisense RNA. For example, in eucaryotes, sense-antisense hybrid formation at the 5'-end non-coding region of an mRNA can be more effective than such a hybrid encompassing the entire coding region. Inouye, Gene 72:25-44 (1988). An antisense RNA that binds solely to the translation initiation site on the target mRNA can be effective, even though such an antisense RNA is typically very short, only a few nucleotides long. Simons, Gene 72:35-44 (1988). In short, it is well within the purview of a person skilled in the art to design a suitable AS_{PSM} locus, based upon a knowledge of the PSM gene selected for use in the genetic construct and upon general principles of antisense RNA action as discussed in a number of reviews on the subject. See, e.g., Simons, id.; Inouye, Gene 72:25-44 (1988); and van der Krol et al., Gene 72:45-50 (1988).

Basically, the positive selection system works as follows: Transformant target cells that have integrated the PSM locus into their genomes will be capable of surviving in an environment selective against cells lacking the PSM locus. That is, transformants possessing a functional and unblocked PSM locus will be

positively selected for when cultured in a selective medium. As stated above, a particularly suitable PSM locus encodes an antibiotic resistance factor that confers upon cells possessing the locus the ability to survive in a culture medium containing the corresponding antibiotic. Any cell lacking an antibiotic-resistance PSM locus, or in which the PSM locus is present but expression thereof is somehow blocked or inhibited, will be killed in medium containing the antibiotic.

The negative selection system works as follows: Transformant cells that have integrated both the PSM and AS loci into their genomes (by non-homologous recombination) will produce antisense RNA that blocks expression of the PSM locus. As a result, the transformants will not produce the corresponding PSM-encoded factor (such as an antibiotic resistance factor) that would otherwise enable the cell to survive in a selective medium. That is, cells possessing the AS_{PSM} and PSM loci (or just the AS_{PSM} locus) are negatively selected against when incubated in selective medium (such as a medium containing the corresponding antibiotic).

As can be seen, both the PSM and AS_{PSM} loci are required in the construct. If the construct lacked the AS_{PSM} locus, then all cells incorporating the construct would be resistant to the antibiotic and would survive. But, there would be no way to selectively distinguish cells that had undergone homologous recombination from cells that had incorporated the construct via non-homologous recombination. The AS_{PSM} locus located outside the region bounded by HFS_1 and HFS_2 provides the necessary negative selection against non-homologous recombinants. This is because homologous recombination of the genetic construct with the genome of the target cell occurs at HFS_1 and HFS_2. Any portions of the genetic construct located outside the region between HFS_1 and HFS_2 are not incorporated into the target cell genome during homologous recombination and are rapidly

degraded. Therefore, in order for the AS_{PSM} locus to negatively select against non-homologous transformants, it must be located outside the region between HFS_1 and HFS_2.

It should be kept in mind that transformed cells that survive incubation in the positive-selection medium (e.g, containing antibiotic) are regarded as "putative recombinants." The surviving subpopulation of putative recombinants must still be screened using conventional techniques (such as Southern blotting) to identify actual (or the best) recombinants. A key advantage of the present invention is that it obviates, for the first time, the necessity to screen the entire population of transformed cells. This is because non-homologously transformed and non-transformed cells (which are not wanted anyway and which represent the majority of the cells) will not survive the positive selection. Thus, the expensive and time-consuming task of screening cells is greatly reduced, at least by several orders of magnitude.

Embodiments Adaptable for Transforming Plant Cells

Genetic constructs as described hereinbelow are particularly adapted to be introduced into plant cells using known plant-cell transformation techniques. The availability of genetic constructs according to the present invention for introduction into plant cells represents a substantial advance because, without exception, all gene targeting experiments performed to date in higher plants exploited positive selectable marker DNA sequences that required exhaustive screening techniques including Southern blotting of all recombinants. Also, there are currently no universally reliable negatively selectable markers available for plant systems. Constructs according to the present invention make it possible, for the first time, to perform targeting of virtually any DNA sequence into plant cells and to readily, conveniently, and inexpensively select for the desired recombinants.

Candidate transformation techniques utilizable with constructs according to the present invention are well known in the art. These include, but are not limited to, utilization of Agrobacterium tumefaciens T-DNA as a vector, electroporation, and particle-gun ("gene gun") bombardment of recipient cells. Use of T-DNA as a vector is a well-known and widely utilized and highly efficient method for delivering DNA into plant cells obtained from dicotyledonous plants. Electroporation and gene gun techniques are more suitable for monocotyledonous plant cells (such as cells from grasses) with which T-DNA is either ineffective or greatly inefficient. However, electroporation and gene gun techniques are less efficient in causing actual integration of the introduced DNA into the target cell genome.

In general, an embodiment of a construct adaptable for transforming plant cells is constructed and works as follows: In the construct, an antibiotic-resistance PSM gene is placed between suitable HFS_1 and HFS_2 loci and an antisense DNA sequence to the PSM is placed outside the region bounded by HFS_1 and HFS_2. A GOI, if desired, is coupled to the PSM locus between HFS_1 and HFS_2. Each of the PSM, GOI (if present), and AS_{PSM} loci are governed by suitable promoters and terminators included in the genetic construct as shown generally in FIG. 1. The construct is administered to target plant cells via a suitable transformation procedure such as Agrobacterium infection. The cells are then cultured in a medium containing the antibiotic. Cells in which a random integration or non-homologous recombination event occurred involving the construct are sensitive to the antibiotic because the antisense DNA sequence will block PSM expression. Cells in which a homologous recombination event occurred are resistant to the antibiotic because only the antibiotic-resistance factor coded by PSM, not additionally the antisense DNA sequence, was integrated into the cell genome. Thus, a

single positive selection agent (the antibiotic) is used to obtain a vastly enriched subpopulation, relative to the population of all target cells, of transformed plant cells that underwent homologous recombination at the desired site or sites in the target cell genome.

By way of example, and not intended to be in any way limiting, one possible general embodiment of a construct suitable for transforming plant cells is shown schematically in FIG 2. The construct shown in FIG. 2 is inherently capable of integrating any of a wide variety of genes of interest (GOI) into dicotyledonous plant cells at predetermined integration sites in the cell genomes. Homologous recombinants resulting from such integration can be readily selected using a single positive selection agent from a large population of cells receiving the construct. The FIG.-2 construct possesses general features of such constructs according to the present invention: (a) a positively-selectable marker DNA sequence such as the NPTII gene encoding neomycin phosphotransferase conferring ability to grow in the presence of kanamycin (the arrow indicates the sense direction); (b) homologous genomic flanking sequences (HFS_1 and HFS_2) having a region therebetween comprising the marker sequence (NPTII), wherein HFS_1 and HFS_2 serve to "target" the genetic construct to integrate at a desired site or sites in the cell genome via homologous recombination; (c) an optional gene of interest (GOI) located adjacent the marker sequence and between HFS_1 and HFS_2, the GOI intended to be integrated into the cell genome at the target site; (d) an antisense DNA sequence to the positively selectable marker sequence (the antisense sequence designated in FIG. 2 as "Anti-NPTII") situated outside the region bounded by HFS_1 and HFS_2; (e) a functional promoter for each of the GOI (if present), NPTII, and Anti-NPTII loci (P1, P_{NOS}, and 35S, respectively); and (f) a functional terminator for each of the GOI (if present), NPTII, and Anti-NPTII loci (T1, T_{NOS}, and 7'5', respectively,

wherein the 7'5' terminator is a strong Agrobacterium terminator known in the art).

The FIG.-2 construct is designed to utilize Agrobacterium tumefaciens T-DNA as a vector. T-DNA is a circular duplex DNA "virulence" plasmid of A. tumefaciens. T-DNA comprises an "RB" (right boundary) locus on one end and a "LB" (left boundary) locus on the opposing end. Published research indicates that the RB and LB loci facilitate integration of T-DNA at various locations in the plant-cell genome and thereby greatly improve the efficiency of plant-cell transformation.

If T-DNA is the vector as shown in FIG. 2, it is apparently necessary that HFS_1 be adjacent the RB locus rather than the LB locus to ensure efficient transformation.

If the plant cells to be transformed are obtained from monocots rather than dicots, use of A. tumefaciens T-DNA is generally not indicated because this bacterium does not efficiently transform monocotyledonous plants. Thus, a genetic construct according to the present invention for use in monocot target cells need not include the RB and LB loci. As discussed above, monocot cells (as well as dicot cells) can be transformed by electroporation or particle-gun bombardment methods known in the art. Other transformation-enhancing methods known in the art can also be employed.

It will be appreciated that a genetic construct according to the present invention can be circular or linear, depending upon the method used to introduce the construct into target cells. For example, use of A. tumefaciens T-DNA requires that the construct be circular (i.e., RB is coupled to LB). With other transformation techniques such as electroporation, a circular construct is not required.

Examples 1-4

In these Examples, four antisense genetic constructs according to the present invention were prepared against the NPTII gene using the transposon Tn5

coding sequence. These constructs are shown schematically in FIG. 3 designated by pCX403, pCX404, pCX407, and pCX408. NPTII is a completely reliable positively selectable marker sequence that is widely used in transformation of plant cells.

In FIG. 3, "RB" and "LB" designate right and left T-DNA borders; "NPTII" designates the neomycin phosphotransferase gene; "Nos-NPTII-Nos" designates a chimeric neomycin phosphotransferase gene controlled by the promoter (left-hand "Nos") and terminator (right-hand "Nos") of the nopaline synthase gene; "35S" is the 35S promoter from cauliflower mosaic virus (CaMV); "7′" and "5′" designate the terminators of gene 7 and gene 5, respectively, of the Ti plasmid; and "rbcS" is the polyadenylation signal of the rubisco ss (rbcS) gene. Arrows indicate the orientation of the nptII sequence.

Construction of the four anti-NPTII constructs began with a 1000-base-pair (1000-bp) SmaI-BglII fragment and a 650-bp NaeI-BglII fragment of Tn5, both including a 35-bp untranslated region and a translation start codon, isolated from pGA342. (pGA342 was a gift from G. An, Washington State University.) To construct pCX403 and pCX404, the 1000-bp and 650-bp fragments were cloned into the Agrobacterium tumefaciens expression binary vector pGA643 at HpaI and BglII sites, respectively, so that the Tn5 coding sequence was in the inverted orientation and under the control of the CaMV 35S promoter.

The pCX407 and pCX408 constructs were generated by inserting the Tn5 coding sequence into the binary vector pKYLX7 (Schardl et al., Gene 61:1-11 (1987)) in the inverted orientation. This was done by first cloning the 1000-bp SmaI-BglII and the 650-bp NaeI-BglII fragments into pBluescript II KS- plasmids at BamHI and EcoRV sites, respectively, thereby creating pCX401 and pCX402, respectively. The Tn5 coding sequences were then released as HindIII-XbaI fragments from pCX401 and pCX402 and inserted into the pKYLX7 expression cassette

in the inverted orientation at corresponding restriction sites to form pCX407 and pCX408.

Examples 5 and 6

In these Examples, the plasmid pBSIIKS-/npt+ (containing the sense nptII gene), the plasmid pBSIIKS-/npt- (containing the anti-nptII gene), and the plasmid pBluescript II KS- (as a control) were used in transient expression assays to determine whether anti-nptII RNA could inhibit the expression of the nptII gene.

To perform these assays, two plasmids, pBSIIKS-/npt+ and pBSIIKS-/npt-, containing the nptII or anti-nptII genes, respectively, were constructed in pBluescript II KS- vectors. To form pBSIIKS-/npt-, an anti-nptII chimeric DNA sequence was isolated from pCX407 as an EcoRI/ClaI fragment and inserted into the pBluescript II KS- vector at corresponding restriction cleavage sites. To form pBSIIKS-/npt+, an NPTII-coding sequence was isolated as a 1000-bp KpnI/XbaI (blunt-ended) fragment from pCX401 and inserted into pKYLX6 (Schardl et al., Gene 61:1-11 (1987)) at blunt-ended KpnI and HindIII sites to allow nptII to be regulated by the CaMV 35S promoter and the terminator of the rbcS gene. The entire cassette, as an EcoRI/ClaI fragment, was inserted into the pBluescript II KS- vector to form pBSIIKS-/npt+.

The transient-expression assays were performed as follows: Protoplasts were isolated from four-day-old tobacco NT-1 cells as described in Michael et al., in Gelvin et al. (eds.), Plant Molecular Biology Manual, Kluwer Academic Publisher, pp. A1/1-A1/16 (1988). Briefly, suspension cells were harvested and incubated in 0.5 M mannitol (pH 5.7) for 30 minutes. The plasmolyzed cells were then digested in an enzyme solution consisting of 0.5% Macerozyme R-10, 2.0% Cellulase R-10, and 0.5 M mannitol, pH 5.7, at 30°C for several hours with gentle shaking on an orbit shaker. Protoplasts were purified by filtering through a 75-μm nylon mesh and washing three times in 0.5 M mannitol (pH

5.7) at 100 x g. Protoplasts were finally resuspended in 0.5 M mannitol (pH 5.7), adjusted to 5 x 10^5 protoplasts/mL, and stored on ice for subsequent electroporation. Electroporation was conducted as described by Saunders et al., Bio/Techniques 7:1124-1131 (1989), using a square-wave pulse generator (Electro Cell Manipulator, Model 600, BTX) and electroporation chambers with 2-mm gaps. The NPTII assay was performed as described in Platt et al., Analytic Biochem. 162:529-535 (1987).

FIG. 4 shows the results of the transient-expression assays. For each assay, 0.5 mL of tobacco protoplasts was mixed with 30 μL DNA solution containing supercoiled plasmid DNA and 50 μg calf-thymus DNA as carrier. Two days after electroporation, protoplasts were collected so that NPTII activity would be assayed. In lane 1, the reaction mixture contained no plasmids (negative control). In lane 2, the mixture contained 20 μg pBluescript II KS- DNA (negative control). In lane 3 (Example 5), the mixture contained 10 μg pBSIIKS-/npt+ DNA plus 10 μg pBSIIKS-/npt- DNA. In lane 4 (Example 6), the mixture contained 10 μg pBSIIKS-/npt+ DNA plus 10 μg pBluescript II KS- DNA. As indicated by Example 5 (lane 3), when plasmids pBSIIKS-/npt+ and pBSIIKS-/npt- in equal molar amounts were co-electroporated into tobacco protoplasts, the NPTII activity was reduced. In contrast, Example 6 (lane 4) indicated that, when plasmid pBSIIKS-/npt+ alone was electroporated into tobacco protoplasts, nptII activity was greatly enhanced. Thus, the presence of the anti-nptII gene in cells caused at least a transient reduction in nptII expression.

Examples 7-17

These Examples comprise biological assays of the pCX403, pCX404, pCX407, and pCX408 constructs of Examples 1-4. The constructs all contained both sense and antisense nptII genes and were used to transform tobacco leaf disks via Agrobacterium-mediated

transformation. The pCX403 construct, containing a full-length anti-nptII gene, and the pCX404 construct, containing two-thirds of the full-length anti-nptII gene, were particularly suitable for an examination of whether the length of the anti-nptII gene had any effect on nptII expression. The pCX407 and pCX408 constructs were used for comparing the efficiency of alternative configurations of the antisense gene and of the transcription terminator. The plasmid pGA643 was employed as a positive control.

The constructs (and pGA643) were separately introduced into Agrobacterium tumefaciens strain EHA105 cells via a freeze-thaw method. An et al., "Binary Vectors," in Gelvin et al. (eds.), Plant Molecular Biology Manual, Kluwer Academic Publishers, pp. A3/1-A3/19 (1988). The intactness of the constructs after introduction into the bacteria was verified by restriction analysis.

Agrobacterium tumefaciens EHA105 cells harboring one of the anti-nptII genetic constructs or pGA643 were used to transform tobacco leaf disks via a co-cultivation method. An et al., id. Briefly, growth-chamber-grown Nicotiana tabacum cv. "Xanthi" plants were grown under a 16-hour photoperiod at 60-percent relative humidity in a walk-in growth chamber. Selected leaves were surface-sterilized in a 1.0% sodium hypochlorite solution. After three subsequent rinses with sterile distilled water, disk-shaped pieces were cut from the leaves using a sterile cork borer. Co-cultivation of the disks with the bacteria was performed in liquid MS medium for three days in the dark. After co-cultivation, the leaf disks were transferred to MS "callusing and shooting medium," as known in the art, containing various concentrations (i.e., 0, 50, 100, 200, or 400 mg/L) of kanamycin sulfate (for selection), and 500 mg/L carbenicillin and 250 mg/L cefotaxime to inhibit agrobacteria. All cultures were maintained under a 16-hour photoperiod at 26°C. The number of

calli that emerged from each leaf disk after four weeks was scored.

Results for Examples 7-11 are shown in FIG. 5, wherein transformed leaf disks were selected on medium containing 200 mg/L kanamycin. Leaf disks transformed by pGA643 (Example 7; positive control) are in the center plate. Leaf disks individually transformed by the four anti-nptII genetic constructs (Examples 8-11) flank the center plate.

Table 1 shows the results of selection of transformed tobacco leaf disks on medium containing various concentrations of kanamycin (Examples 12-17). In Table 1, entries marked "+++" indicate that calli formed all around the cut edges of the leaf disks (more than 20 calli per leaf disk). Numerical values represent the average of two similar experiments. Each experiment employed three replica plates for each concentration of kanamycin, with four leaf discs per plate.

Table I

Example	Construct	Kanamycin Concentration (mg/L) 0	50	100	200	400
12	pGA643	+++	+++	+++	+++	+++
13	pCX403	+++	4.50	2.45	0.00	0.00
14	pCX404	+++	4.50	2.45	0.00	0.00
15	pCX407	+++	4.51	2.55	0.00	0.00
16	pCX408	+++	4.20	2.15	0.00	0.00
17	none	+++	0.00	0.00	0.00	0.00

In Example 12, pGA643 served as the positive control. Example 17 was a negative control that lacked any genetic constructs.

As can be seen in these Examples, the presence of any of the four antisense constructs in leaf-disk cells completely inhibited callus and shoot formation when the disks were cultured on media containing 200 mg/L kanamycin. The same inhibition was observed at higher kanamycin concentrations in cells containing antisense constructs, as shown in Table 1. Table 1 also shows that kanamycin concentrations lower than 200 mg/L did not completely inhibit callusing or shoot formation by cells containing antisense constructs. Thus, the kanamycin-resistance phenotype can be overcome by antisense RNA if sufficient antibiotic is supplied to the selection medium.

These Examples also illustrated that anti-NPTII genes are completely effective as negative-selection markers with clear utility for gene targeting according to the present invention.

These Examples also demonstrated that a genetic construct comprising the NPTII sequence as a PSM and an anti-NPTII locus can function as a positive/negative

selectable marker. The assay employed in these Examples allowed the appropriate kanamycin concentration required for negative selection to be determined by comparing behavior of leaf disks containing antisense constructs to positive controls. The assay also exemplified methods that can be used or adapted by persons skilled in the art to construct any of a wide variety of genetic constructs according to the present invention.

Examples 18-19

Since the anti-nptII constructs used in Examples 7-17 prevented the recovery of stable transformants, the present Examples were a confirmation of the "antisense RNA effect." That is, the present Examples are a confirmation that the results of Examples 7-17 were not due to an artifact of intra-molecular homologous recombination that could possibly lead to inversion for pCX403 and pCX404 and deletion for pCX407 and pCX408.

In these Examples, the plasmid pCX407/hpt was constructed, as shown in FIG. 6, wherein "35S" designates the CaMV 35S promoter; "3'rbcS" designates the polyadenylation signal of the rbcS gene; "nptII" designates the neomycin phosphotransferase gene; "Nos-nptII-Nos" designates the nptII DNA sequence regulated by the promoter and terminator of the nopaline synthase gene; "35S-hpt-Nos" designates the hygromycin-resistance gene regulated by the 35S promoter and the terminator of the nopaline synthase gene; and the bold arrows indicate the orientation of the nptII sequence. FIG. 6 also shows the pKYLX7 plasmid as described in Schardl et al., Gene 61:1-11 (1987).

The pCX407/hpt plasmid was constructed by modifying the pCX407 construct to include a hygromycin-resistance gene. The hygromycin-resistance gene, regulated by the CaMV 35S promoter and the polyadenylation signal of the nopaline synthase gene, was isolated from the plasmid pGA883 (gift from G. An of Washington State University) as an XbaI fragment. The ends of the XbaI fragment were blunted by treating with T4 DNA polymerase (Promega),

and the fragment was inserted into pCX407 at a ClaI site which had been blunt-ended in the same way. E. coli strain DH10B was used for all cloning.

The pCX407/hpt plasmid (Example 18), or the pKYLX7 plasmid (Example 19) used as a control, was introduced into cells of Agrobacterium tumefaciens strain LBA4404. The bacteria were subsequently used to transform tobacco leaf disks as previously described. Individual hygromycin-resistant calli that arose were maintained on hygromycin-containing MS "callusing" medium. To ascertain kanamycin sensitivity, hygromycin-resistant calli were transformed to MS "callusing" medium containing 200 mg/L kanamycin. Seven hygromycin-resistant calli were found to be inhibited on kanamycin-containing medium as evidenced by zero gain in fresh callus weight (data not shown). These results indicated that the introduced anti-nptII sequences are functional in transformed calli and are able to reduce NPTII activity. These results also showed that these anti-nptII constructs have general utility for negative selection.

Examples 20-24

These Examples constitute an examination of whether, in Examples 7-17, it was the anti-nptII sequence that caused the observed loss or decrease in kanamycin resistance. In these Examples, four representative calli that were sensitive to kanamycin were chosen for further study by Southern-blot analysis as known in the art.

To prepare for Southern blotting, total DNA was isolated from callus tissue by the CTAB method. Ausubel et al., Current Protocols in Molecular Biology, Wiley (1987). Nucleic-acid concentrations were quantified spectrophotometrically.

For Southern blotting, 10 μg of total DNA from each callus was digested with EcoRI and BamHI, fractionated on a 1.0% agarose gel, and blotted onto a nitrocellulose filter via capillary transfer. BstEII-digested λDNA was

used as DNA size markers. A digoxigenin-labeled probe (FIG. 6) was prepared using a random-primer labeling and detection kit (Boehringer-Mannheim). Hybridization was carried out in 5 x SSC at 65°C in a rotary hybridization oven. Filters were washed twice at room temperature for 15 minutes each in 2 x SSC with 0.1% SDS, then in 0.1 x SSC with 0.1% SDS, and finally in 0.1 x SSC with 0.1% SDS at 65°C for 15 minutes.

Results of the Southern blotting experiments are shown in FIG. 7, wherein BstEII-digested λ-DNA size markers are shown on the left, lane c represents the control (Example 20), and lanes 1-4 correspond to individual transformed calli (Examples 21-24, respectively).

As shown in FIG. 7, all the kanamycin-sensitive calli of Examples 21-24 (lanes 1-4, respectively) contained a DNA fragment of 2.3 kb and another fragment of 3.4 kb which hybridized to the probe, indicating the integration of both the nptII and the anti-nptII sequences. The control callus of Example 20 (lane c) transformed with pKYLX7 alone only contained a DNA fragment of 2.7 kb, indicating the presence of the nptII sequence.

Examples 25-29

In these Examples, Northern blot analysis, as well known in the art, was performed to estimate the degree of expression of the sense and antisense nptII sequences.

Total RNA was isolated from transformed calli and control calli using a guanidium isothiocyanate procedure, Chirgwin et al., Biochemistry 18:5296 (1979), and using an RNA Isolation Kit (Promega). For each assay, 10 μg total RNA was fractionated on a 1.0% formaldehyde-agarose gel and transferred onto a nitrocellulose membrane. Manufacturer's instructions for hybridization and detection were followed.

Sense and antisense probes were generated via a modified asymmetric PCR method (McCabe, in Innis et al.

(eds.), PCR Protocols: A Guide to Methods and Applications, Academic Press, pp. 76-83 (1990)) using a single primer and the pCX401 plasmid (in linearized form and containing the nptII insert) as the template. Briefly, pCX401 was linearized with either HindIII (to make an antisense probe) or KpnI (to make a sense probe). The reaction mixture was prepared according to standard PCR methods except that only one primer and digoxigenin-11-dUTP (Boehringer-Mannheim) were included. The SK primer (Stratagene) was used for generating the sense probe and the KS primer was used for generating the antisense probe.

Two duplicate filters were prepared (FIGS. 8A and 8B). One filter (FIG. 8A) was probed with the digoxigenin-labeled sense probe and the other (FIG. 8B) with the digoxigenin-labeled antisense probe. In both FIGS. 8A and 8B, lane c is a control (Example 25), and lanes 1-4 represent individual transformed calli (Examples 26-29, respectively). In the control calli (lane c of Figs. 8A and 8B), only sense transcripts of nptII were detectable at high levels; antisense transcripts were not detected. In calli sensitive to kanamycin (lanes 1-4), both sense and antisense transcripts were detected, but the level of sense transcripts was greatly reduced compared with the level of sense transcripts in control calli (Fig. 8A). The level of antisense transcripts in kanamycin-sensitive calli was high (FIG. 8B). These data indicated that inhibition of NPTII is at the post-transcriptional level.

Example 30

Another example of a GOI is the acyl-carrier protein (ACP-I) from Arabidopsis. Acyl-carrier protein is a co-substrate and cofactor in de novo lipid synthesis in plants. The NPTII sequence, as an example of a suitable PSM, can be inserted between two genomic sequences flanking the ACP-I gene in Arabidopsis. (This is possible because we have cloned the ACP-I gene and

have subclones that lack the coding sequence but have flanking sequences.) The NPTII sequence is also inserted between the flanking sequences. The antisense NPTII sequence is placed immediately outside the ACP-I flanking regions to create pHRI, shown in FIG. 9. (In FIG. 9, the dark regions designate Arabidopsis ACP-I homologous flanking sequences.)

Subsequent co-cultivation of target cells with Agrobacteria containing the pHRI construct is followed by selection on kanamycin-containing medium. Calli resistant to the antibiotic are subcultured to regenerate plants from surviving homologously recombinant cells. Southern-blot analysis can be performed on selected transformants (not the entire population of target cells!) by probing with intact ACP-I gene. Any band shifts seen on the blots with individual transformants demonstrate that the selection procedure has disrupted the genomic target allele via homologous recombination. Such fragments can be gel-purified using conventional techniques. Polymerase chain reaction (PCR) methods can also be used to verify the disruption. (For PCR, the sense NPTII sequence is used as the template.)

Having illustrated and described the principles of our invention in several preferred embodiments, examples, and variations thereof, it should be apparent to those skilled in the art that the present invention may be modified in arrangement and detail without departing from said principles. We claim as our invention all modifications coming within the scope and spirit of the following claims.

CLAIMS

1. A genetic construct comprising a sense DNA sequence gene for a positively selectable genetic trait and an antisense DNA sequence coupled to the sense DNa sequence, wherein the antisense DNA sequence is antagonistic to expression of the sense DNA sequence.

2. A genetic construct as recited in claim 1 wherein the sense DNA sequence encodes an antibiotic resistance factor.

3. A genetic construct as recited in claim 1 adapted for transforming cells by homologous recombination.

4. A genetic construct as recited in claim 3 further comprising first and second flanking sequences homologous to sequences flanking an integration site on DNA from said cells, wherein the sense DNA sequence is between the first and second homologous flanking sequences and the antisense DNA sequence is not between the first and second homologous flanking sequences.

5. A genetic construct as recited in claim 4 further comprising a gene of interest located adjacent the sense DNA sequence and between the first and second homologous flanking sequences.

6. A genetic construct adapted to integrate into a target DNA by homologous recombination, the construct comprising:

(a) first and second homologous flanking sequences homologous with genetic sequences flanking a desired integration site in the target DNA, the homologous flanking sequences capable of undergoing homologous recombination with said genetic sequences flanking the desired integration site;

(b) a positively selectable genetic marker situated in a portion of the construct located between the first and second homologous flanking sequences so as to enable the positively selectable genetic marker to become integrated into said target DNA when the

construct homologously recombines with the target DNA; and

(c) an antagonistic DNA sequence that inhibits expression of the positively selectable marker, the antagonistic DNA sequence being situated outside the region located between the first and second homologous flanking sequences.

7. A genetic construct as recited in claim 6 adapted to integrate into a plant-cell genome.

8. A genetic construct as recited in claim 6 adapted to integrate into an animal-cell genome.

9. A genetic construct as recited in claim 6 wherein the antagonistic DNA sequence is adapted to integrate into the target DNA whenever the genetic construct non-homologously recombines with the target DNA but not when the genetic construct homologously recombines with the target DNA.

10. A genetic construct as recited in claim 6 wherein the positively selectable genetic marker encodes an antibiotic resistance factor.

11. A genetic construct as recited in claim 6 wherein the antagonistic DNA sequence is an antisense DNA sequence to the positively selectable marker.

12. A genetic construct as recited in claim 6 further comprising a gene of interest situated adjacent the positively selectable marker between the first and second homologous flanking sequences.

13. A genetic construct as recited in claim 12 wherein the gene of interest comprises a portion of a gene.

14. A genetic vector including the construct of claim 6.

15. An infectious agent that includes the genetic vector of claim 14.

16. A target DNA that has undergone homologous recombination with the construct of claim 6 so as to incorporate the positively selectable genetic marker into the target DNA.

17. A target DNA as recited in claim 16 that is a genome.

18. A cell having the genome of claim 17.

19. An organism generated from the cell of claim 18.

20. A cell having the target DNA of claim 16.

21. An organism generated from the cell of claim 20.

22. A genetic construct adapted to transform a target cell by homologously recombining with genomic DNA of the target cell, the genetic construct comprising:

(a) first and second homologous flanking sequences homologous with genetic sequences flanking a desired integration site in the genomic DNA, the homologous flanking sequences capable of undergoing homologous recombination with said genetic sequences flanking the desired integration site;

(b) a positively selectable genetic marker located in a region situated between the first and second homologous flanking sequences wherein the positively selectable genetic marker is adapted to integrate into the genomic DNA whenever the construct homologously recombines with the genomic DNA; and

(c) an antisense gene capable of preventing expression of the positively selectable marker, the antisense gene located outside the region situated between the first and second homologous flanking sequences so as to enable the antisense gene to integrate into the genomic DNA whenever the construct non-homologously recombines with the genomic DNA but not when the construct homologously recombines with the genomic DNA.

23. A construct as recited in claim 22 further comprising a gene of interest located adjacent the positively selectable marker in the region between the first and second homologous flanking sequences so as to allow the gene of interest to become integrated into the

genomic DNA whenever the construct homologously recombines with the genomic DNA.

24. A construct as recited in claim 23 further comprising at least one promoter for the positively selectable genetic marker and the gene of interest, the promoter being located in the region between the first and second homologous flanking sequences.

25. A construct as recited in claim 22 further comprising a separate promoter for the positively selectable genetic marker and the antisense gene.

26. A construct as recited in claim 25 further comprising a separate terminator for the positively selectable genetic marker and the antisense gene.

27. A construct as recited in claim 22 wherein the positively selectable genetic marker encodes an antibiotic resistance factor.

28. A construct as recited in claim 22 adapted to transform a plant cell.

29. A construct as recited in claim 22 adapted to transform an animal cell.

30. A genetic vector including the construct of claim 22.

31. An infectious agent including the genetic vector of claim 30.

32. A genome that has undergone homologous recombination with the construct of claim 22 so as to incorporate the positively selectable genetic marker into the genome.

33. A target cell having the genome of claim 32.

34. An organism generated from the target cell of claim 33.

35. A method for transforming target cells, comprising:

(a) providing target cells comprising target-cell DNA having first and second recombinatable regions flanking an integration site;

(b) providing a genetic construct adapted to recombine with the target DNA, the genetic construct

comprising a sense DNA sequence encoding a positively selectable marker; first and second flanking sequences homologous to the first and second recombinatable regions, respectively, in the target DNA, wherein the sense DNA sequence is located between the first and second flanking sequences; and an antisense DNA sequence not located between the first and second flanking sequences, wherein the antisense DNA sequence is adapted to block expression of the sense DNA sequence;

(c) delivering the genetic constructs of step (b) inside a population of the target cells of step (a);

(d) in each target cell of step (c), allowing the first and second recombinatable regions of the target DNA to recombine inside the target cell with the first and second flanking sequences, respectively, of the genetic construct, thereby forming a subpopulation of homologously recombinant cells having, integrated into the target DNA at the integration site, the sense DNA sequence but not the antisense DNA sequence; a subpopulation of non-homologously recombinant cells having both the sense DNA sequence and the antisense DNA sequence integrated into the target DNA; and a subpopulation of non-recombinant cells; and

(e) exposing the cells to a selection agent such that only the subpopulation of homologously recombinant cells survives the exposure.

36. A method for transforming target cells each having a target cell genome comprising an integration site flanked by first and second recombinatable regions, the method comprising:

(a) providing a genetic construct capable of transforming the target cells by homologous recombination, the construct comprising a sense gene expressible in the target cells and encoding a capacity to survive exposure to a positive-selection agent; first and second flanking sequences homologous to the first and second recombinatable regions, respectively, wherein

the sense gene is situated between the first and second flanking sequences; and an antisense gene expressible in the target cells but not situated between the first and second flanking sequences, the antisense gene being capable of blocking expression of the sense gene;

(b) delivering the genetic construct inside the target cells;

(c) allowing the genetic construct delivered inside the target cells to recombine with the target cell genomes so as to form homologously recombinant cells that have integrated the sense gene but not the antisense gene at the integration site, non-homologously recombinant cells that have integrated both the sense gene and the antisense gene into their genomes, and non-recombinant cells; and

(d) exposing the cells to the positive selection agent so as to selectively kill non-homologously recombinant cells and non-recombinant cells but not homologously recombinant cells.

37. A method as recited in claim 36 wherein the positive selection agent is an antibiotic and step (d) comprises culturing the cells in a medium comprising the antibiotic.

38. A method for transforming target cells each having a target cell genome comprising an integration site flanked by first and second recombinatable regions, the method comprising:

(a) providing a genetic construct capable of transforming the target cells by homologous recombination, the construct comprising a sense gene expressible in the target cells and encoding a factor necessary for cells to survive exposure to a positive-selection agent; a gene of interest adjacent the sense gene; first and second flanking sequences homologous to the first and second recombinatable regions of the target cell genome, respectively, wherein the sense gene and the gene of interest are situated between the first and second flanking sequences; and an antisense gene

expressible in the target cells but not situated between the first and second flanking sequences, the antisense gene being capable of blocking expression of the sense gene;

(b) delivering the genetic construct inside the target cells;

(c) allowing the genetic constructs delivered inside the target cells to recombine with the target cell genomes so as to form homologously recombinant cells that have integrated the sense gene and the gene of interest but not the antisense gene at the integration site, non-homologously recombinant cells that have integrated both the sense gene and the antisense gene into their genomes, and non-recombinant cells; and

(d) exposing the cells to the positive selection agent so as to selectively kill non-homologously recombinant cells and non-recombinant cells but not homologously recombinant cells.

39. A method as recited in claim 38 wherein the positive selection agent is an antibiotic and step (d) comprises culturing the cells in a medium containing the antibiotic.

FIG. 1

FIG. 2

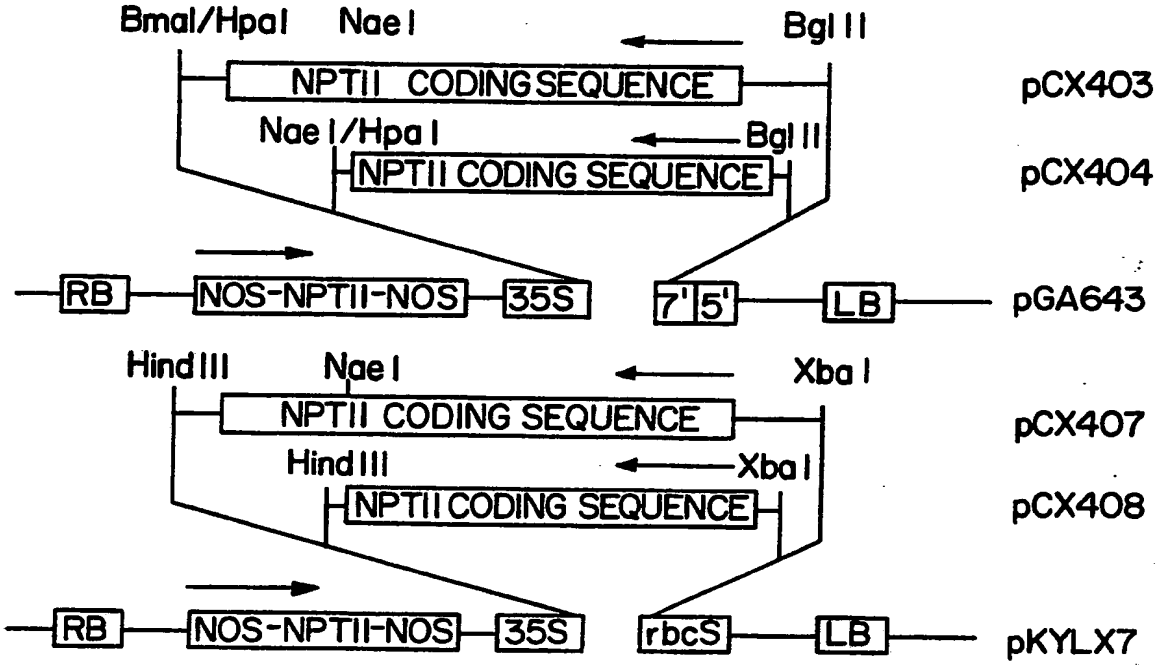

BmaI/HpaI
NaeI
BglII
NPTII CODING SEQUENCE
pCX403
NaeI/HpaI
BglII
NPTII CODING SEQUENCE
pCX404
RB
NOS-NPTII-NOS
35S
7'
5'
LB
pGA643
HindIII
NaeI
XbaI
NPTII CODING SEQUENCE
pCX407
HindIII
XbaI
NPTII CODING SEQUENCE
pCX408
RB
NOS-NPTII-NOS
35S
rbcS
LB
pKYLX7

FIG. 3

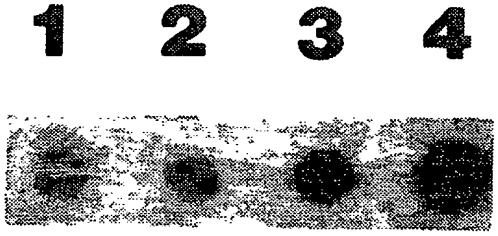


FIG. 4

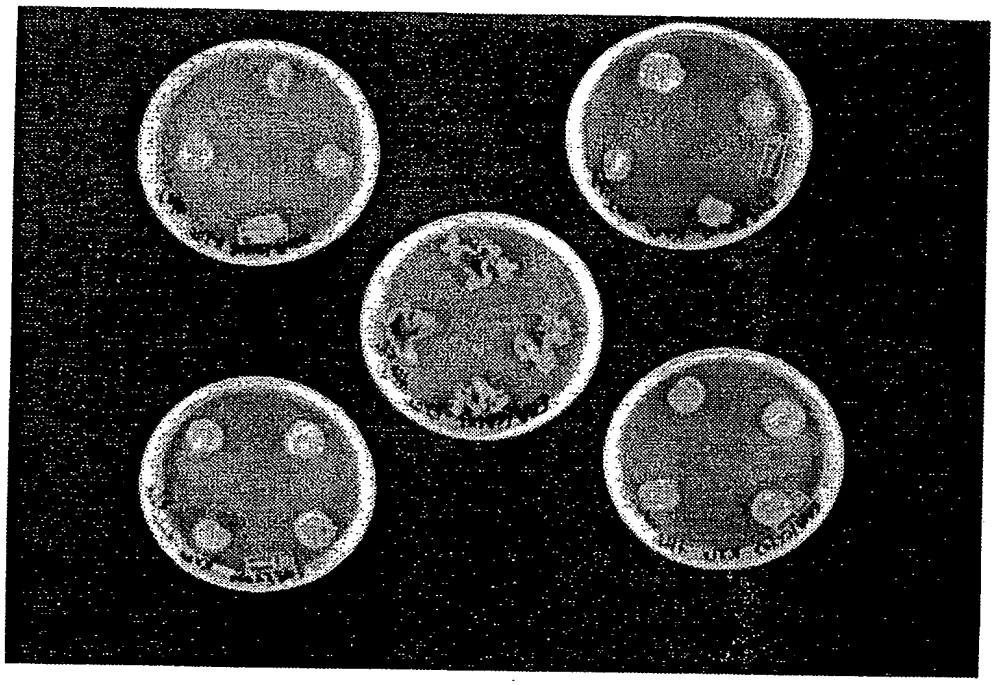


FIG. 5

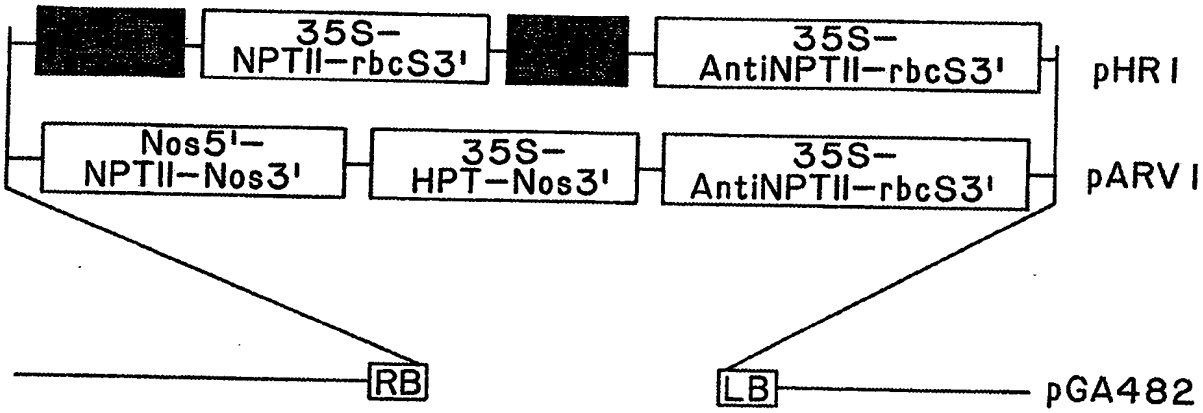
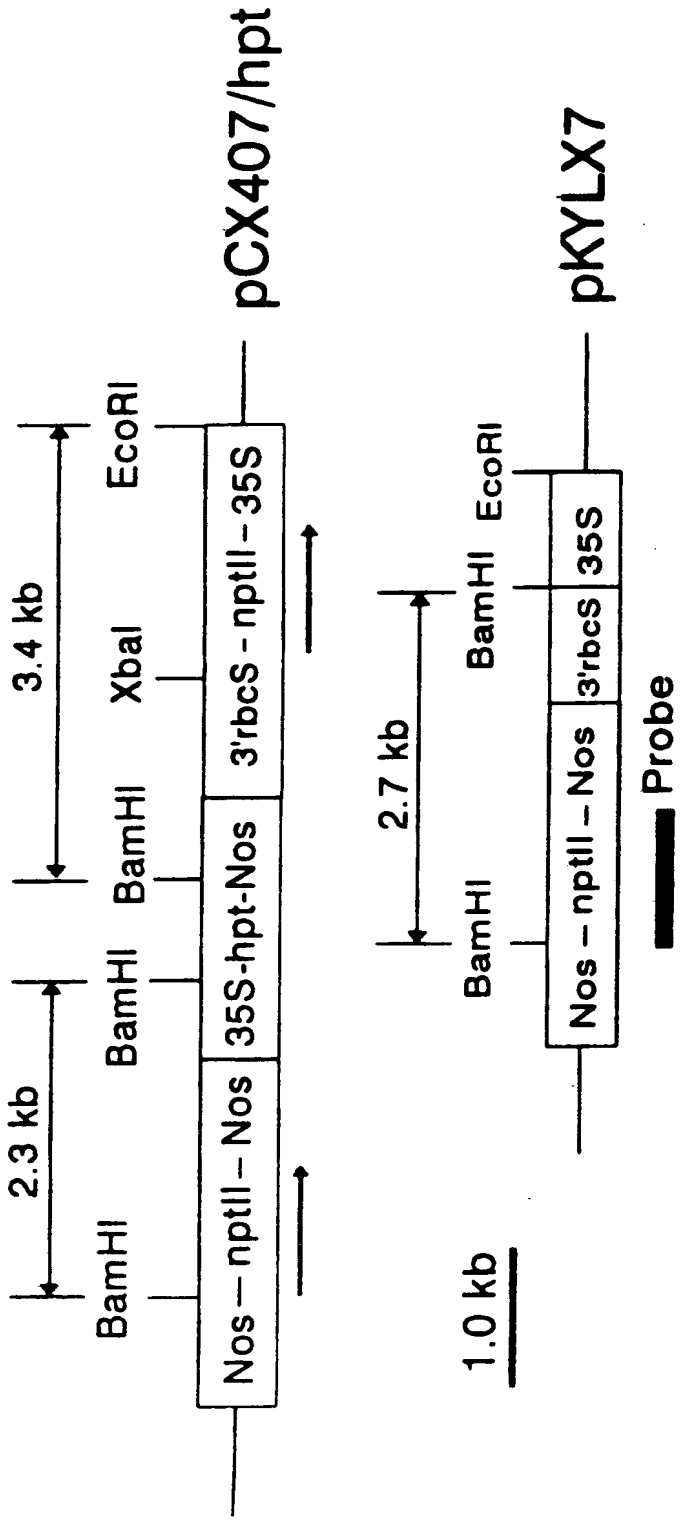


FIG. 9

FIG. 6



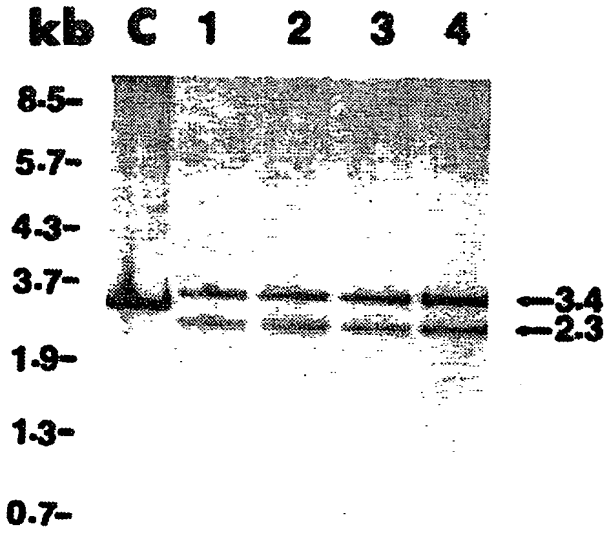


FIG. 7

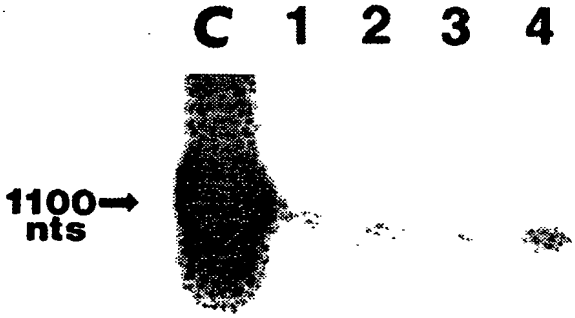


FIG. 8A

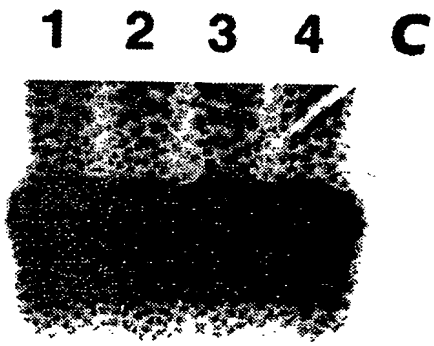


FIG. 8B

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/08513

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/11; C12N15/90; C12N15/82; C12N5/10
A01H5/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A01H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	WO,A,9 008 828 (PALADIN HYBRIDS) 9 August 1990 see example 21	1,2
X	EP,A,0 240 208 (CALGENE) 7 October 1987 see page 6, line 1 - line 18	1,2
X	WO,A,9 102 070 (MOGEN) 21 February 1991 see the whole document	16-21, 32-34

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
28 JANUARY 1993	[illegible] 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MADDOX A.D.

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9208513
SA 65693

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 28/01/93

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9008828	09-08-90	AU-A-	5037290	24-08-90
		EP-A-	0456706	21-11-91
		JP-T-	4504355	06-08-92
EP-A-0240208	07-10-87	AU-A-	1301792	03-09-92
		AU-B-	618234	19-12-91
		AU-A-	7059787	01-10-87
		EP-A-	0458367	27-11-91
		JP-A-	62296880	24-12-87
		US-A-	5107065	21-04-92
		US-A-	4801540	31-01-89
WO-A-9102070	21-02-91	NL-A-	8901931	18-02-91
		EP-A-	0436007	10-07-91
		JP-T-	4502860	28-05-92